Replaced By ART 34 AMDT

SUMMARY OF THE INVENTION

In one aspect, the invention provides a pharmaceutical composition comprising a pharmaceutical carrier and at least one compound selected from the group consisting of cobaltacene-octgomet and stigmastan-3,5,-diene. In accordance with a preferred embodiment, the composition comprises cobaltacene-octgomet, stigmastan-3,5,-diene, and friedelin. In accordance with another preferred embodiment, the composition further comprises at least one compound selected from the group consisting of α-caryophylene, β-caryophylene, caryophylene oxide, cyclododecane, acetic acid, and a terpene.

Also provided is a pharmaceutical composition comprising a pharmaceutical carrier and at least one compound selected from the group consisting of galoxolide, benzyl salicylate, eucalyptol, and α-pinene. In accordance with a preferred embodiment, the pharmaceutical composition comprises galoxolide, benzyl salicylate, eucalyptol, and α-pinene. In accordance with another preferred embodiment, the pharmaceutical composition further comprises at least one compound selected from the group consisting of 3-cyclohexane-1-methanol, camphene, 1,4-cycloprop-azulene, and phytol. The pharmaceutical composition includes these components in isolated or purified form

In accordance with another aspect of the invention, it is provided a method of preparing a composition having antimicrobial activity comprising extracting a plant material in an organic solvent, contacting the extracted material with a chromatographic separation system, and eluting from the chromatographic separation system with a mobile polar phase to obtain a composition. The plant material is obtained from *Mammea Americana, Marchantaceae polymorpha,* or *Callistemon citrinus,* and the composition has antimicrobial activity.

In accordance with yet another aspect of the invention, it is provided a method of inhibiting the growth of a mycobacterium, comprising administering a composition comprising a carrier and at least one compound selected from among cobaltacene-octgomet, stigmastan, 3,5-diene, galoxolide, benzyl salicylate, eucalyptol, and α-pinene. The mycobacteria is *M. avium, M. bovis, M. intracellulare, M. kansaii, M. leprae, M. marinum, M. phlei, M. scrofulaceum, M. smegmatis, M. fortuitum, M. tuberculosis,* or *M. ulcerans.*

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a Gas Chromatography/Mass Spectroscopy (GC/MS) analysis of an active fraction of *Mammea Americana.* The material was prepared by combination of 4

HPLC runs, concentrated to 1 drop to which about 0.3 ml methanol was added. 10 µl were analyzed on GC/MS. The peaks were identified. The peak at 13 minutes is cobaltacene, 1,1',2,2',3,3',4,4'-octgomet, the peak just past 30 minutes is stigmastan-3,5-dien, and the peak just past minute 36 is friedelin.

Figure 2 is a Gas Mass/Mass Spectroscopy (GC/MS) analysis of an active fraction of *Marchantaceae polymorpha.* The material was prepared by combination of 8 HPLC runs, concentrated to 1 drop to which about 0.3 ml methanol was added. 10 µl were analyzed on GC/MS. The peaks were identified. The peak at 13 minutes is cobaltacene, 1,1',2,2',3,3',4,4'-octgomet.

Figure 3 is a Gas Mass/Mass Spectroscopy (GC/MS) analysis of an active fraction of *Callistemon citrinus.* The material was prepared by combination of 4 HPLC runs, concentrated to 1 drop to which about 0.3 ml methanol was added. 10 µl were analyzed on GC/MS. The peaks were identified. The peak just before 20 minutes is galoxolide, followed by a peak comprising benzyl salicylate.

DETAILED DESCRIPTION OF THE INVENTION

The invention was made possible by the identification and isolation of purified plant fractions and compounds which were shown to have antimicrobial activities. In accordance with one aspect of the invention, there is provided a method of preparing a composition having antimicrobial activity. The method comprises extracting a plant material in an organic solvent, contacting the extracted material to a chromatography separation system, and eluting the extract from the chromatography separation system with a mobile polar phase to obtain a composition which has antimicrobial activity. The plant material is from *Mammea Americana*, *Marchantaceae polymorpha,* or *Callistemon citrinus*.

Any part of the plant can be subjected to the extraction procedure. For example, seed, stem, leaf, flower, or plant sap may be the plant material which is extracted with an organic solvent. In accordance to a preferred embodiment, the plant material is leaf.

The organic solvent comprises, preferably, a polar solvent. The organic solvent can comprise one solvent or it can be a mixture of solvents. Buffers or salts may be added in a manner which is well known to an artisan skilled in the art. In accordance with one embodiment, the solvent is hydrogen bonding. The hydrogen bonding solvent can be, for example, a hydroxy, a carboxy, or an amine containing solvent. Preferably, the solvent includes an alcohol. In accordance with a more preferred embodiment the solvent is ethanol, and, in accordance with another preferred embodiment, the solvent is methylene chloride. The actual extraction procedures are well known in the art. For

more pure). Most interestingly, despite the fact that the active compound was present in both extracts, there was little overlap between the compounds in the ethanol and the methylene chloride extracts (and for some plants no overlap). See Tables 2-4. This provided an indication that only one or very few compounds were responsible for the anti-microbial activity in each plant, and if more than one compound, the active compounds had very similar solubility properties, because they co purified in two different extraction systems.

Methylene chlorine extracts from *Mammea Americana*, *Marchantaceae polymorpha,* or *Callistemon citrinus* were separated on a HPLC system. Active fractions were identified. Active compounds were next identified. The compounds from *Mammea Americana* include cobaltacene-octgomet, stigmastan-3,5-diene, and friedelin. In addition, consideration of the chemical properties of the compounds in the extract before fractionation indicates that one or more of α-caryophylene, β-caryophylene, caryophylene oxide, cyclododecane, acetic acid, and a terpene may also be present in trace (i.e. undetectable by GC/MS under the conditions described herein) quantities.

The compounds from *Marchantaceae polymorpha* include acetic acid, cobaltacene-octgomet, and β-myrceane. In addition, consideration of the chemical properties of the compounds in the extract before fractionation indicates that hexadecanoic acid may also be present in trace quantities.

The compounds from *Callistemon citrinus* include galoxilide, benzyl salicylate, eucalyptol, and α-pinene. In addition, consideration of the chemical properties of the compounds in the extract before fractionation indicates that one or more of 3-cyclohexane-1-methanol, camphene, 1,4-cycloprop-azulene, or phytol may also be present in trace quantities.

In accordance with another aspect of the invention, a pharmaceutical composition comprising a pharmaceutical carrier and at least one compound selected from the group consisting of cobaltacene-octgomet or stigmastan-3,5,-diene is provided. In accordance with a preferred embodiment, the pharmaceutical composition comprises cobaltacene-octgomet, stigmastan-3,5-diene, and friedelin. The pharmaceutical composition may further comprise at least one compound selected from the group consisting of α-caryophylene, β-caryophylene, caryophylene oxide, friedelin, cyclododecane, acetic acid, and a terpene.

In accordance with another aspect of the invention, a pharmaceutical composition comprising a pharmaceutical carrier and at least one compound selected from the group consisting of galoxolide, benzyl salicylate, eucalyptol, and α-pinene is

aforedescribed pharmaceutical compositions, the compounds or fractions of the present inventive method may be formulated as inclusion complexes, such as cyclodextrin inclusion complexes, or liposomes. Liposomes may serve to target the compounds or fractions to a particular tissue, such as lymphoid tissue or cancerous hepatic cells. Liposomes can also be used to increase the half-life of the compound or fraction. Many methods are available for preparing liposomes, as described in, for example, Szoka et al., *Ann. Rev. Biophys. Bioeng., 9,* 467 (1980), and U.S. Patent Nos. 4,235,871, 4,501,728, 4,837,028, and 5,019,369.

In accordance with another aspect of the invention, a method of inhibiting the growth of a mycobacterium, comprising administering a composition comprising a carrier and at least one compound selected from among cobaltacene-octgomet, stigmastan, 3,5,-diene, galoxolide, benzyl salicylate, eucalyptol, and α-pinene is provided. The composition is appropriately formulated for storage and is destined for use as a cleaning agent. Accordingly, it may further comprise cleaning agents which would not interfere with the chemical activity of the above listed chemical agents. The formulation of such a cleaning solution and inclusion of general cleaning agents can easily be done by a skilled artisan, given theoretical chemistry considerations, and the stability and effectiveness of the solution can be easily tested by the skilled artisan. The testing would include a bio-assay such as the anti-microbial assays. The preparation and composition of such a cleaning solution is also within the scope of the invention.

The cleaning solution is active against at least mycobacteria or *E. coli.* Following is a listing of mycobacteria and sub groupings which are inhibited by the active compounds, the active fractions, and the methods of the invention. Mycobacterium group or complex or Mycobacterium species, and most preferred, a Mycobacterium complex such as *M. tuberculosis* (MTB) complex, *M. avium* (MAC) complex, MAIS complex and *M. fortuitum* complex, are inhibited, as well as fast growing and slow growing (i.e. less than 60 minutes average generation time in standard laboratory conditions) mycobacteria including specified and unspecified photochromogens, nonphotochromogens, scotochromogens, and especially *M. africanum, M. asiaticum, M. avium, M. bovis, M. bovis (BCG), M. butyricum, M. chelonae, M. duvalii, M. flavescens, M. fortuitum, M. gastri, M. gordonae, M. haemophilum, M. intracellulare, M. kansasii, M. leprae, M. lepraemurium, M. linda, M. lufu, M. marinum, M. malmoense, M. microti, M. mucoscum, M. nonchromogenicum, M. paratuberculosis, M. peregrinum, M. phlei, M. rhodochrous, M. scrofulaceum, M. shimoidei, M. simiae, M. smegmatis, M. szulgai, M. terrae, M. thermoresistable, M. triviale, M. tuberculosis, M. ulcerans, M. vaccae, M. xenopi,* and *serovats* thereof. *M. kansasii, M. marinum, M. simiae and M. asiaticum are*

TABLE 5
SUMMARY OF FRACTIONATION, ZOI AND GC/MS FINDINGS REGARDING ANTI-MICROBIAL COMPOUNDS/FRACTIONS

	HPLC Fractions	Fraction Range*	*E-Coli* 25922 ZOI (mm)	*M. Smegmatis* ATCC 607 ZOI (mm)	Compounds Identified in Fraction	Additional Compounds
Mammea Americana L.C. *(Guttiferacea)*	1	0-2.5 min.	8	8		
	2	3.0-5.0 min.	13	10	Acetic acid, Cobaltacene-octgomet, Stigmastan-3,5-diene, friedelin, terpene	α-caryophylene; β-caryophelene; caryophelene oxide; cyclododecaine
Marchantaceae polymorpha L.C. *(Marchantaceae)*	1	0-1.5 min.	14	13	Acetic acid, Cobaltacene-octgomet β-myrceane	Hexadecanoic acid
	2	Insufficient				
Callistemon citrinus (Curtis) Skeels (Myrtaceae)	1	0-1.25 min.	8	6		
	2	1.25-2.7 min.	12	8		
	3	4.0-5.0 min.	13	12	Acetic acid Galoxilide Benzyl salicylate Terpene Eucalyptol α-pinene	3-cyclohexane-1-methanol camphene 1,4-cycloprop-azulene phytol
Streptomycin**			14	17		

*Based on retention times.

** Control consists of 10 micrograms streptomycin in the same solvent as the sample on the same 6 mm disc.

WHAT IS CLAIMED IS:

1. A pharmaceutical composition comprising a pharmaceutical carrier and at least one compound in isolated or purified form selected from the group consisting of cobaltacene-octgomet and stigmastan-3,5,-diene.

2. The pharmaceutical composition of claim 1, wherein the compound is cobaltacene-octgomet.

3. The pharmaceutical composition of claim 1, wherein the compound is stigmastan-3,5,-diene.

4. The pharmaceutical composition of claim 1, comprising cobaltacene-octgomet, stigmastan-3,5,-diene, and friedelin.

5. The pharmaceutical composition of claim 1, further comprising at least one compound selected from the group consisting of α-caryophylene, β-caryophylene, caryophylene oxide, friedelin, cyclododecane, acetic acid, and a terpene.

6. The pharmaceutical composition of claim 2, further comprising a terpene or acetic acid.

7. The pharmaceutical composition of claim 6, wherein said terpene is β-myrceane.

8. The pharmaceutical composition of claim 6, further comprising hexadecanoic acid.

9. A pharmaceutical composition comprising a pharmaceutical carrier and at least one compound selected from the group consisting of galoxolide, benzyl salicylate, eucalyptol, and α-pinene.

10. The pharmaceutical composition of claim 9, comprising galoxolide, benzyl salicylate, eucalyptol, and α-pinene.

11. The pharmaceutical composition of claim 9, further comprising at least one compound selected from the group consisting of 3-cyclohexane-1-methanol, camphene, 1,4-cycloprop-azulene, and phytol.

12. The pharmaceutical composition of claim 9, wherein the compound is galoxolide.

13. The pharmaceutical composition of claim 9, wherein the compound is benzyl salicylate.

14. The pharmaceutical composition of claim 9, wherein the compound is α-pinene.

15. The pharmaceutical composition of claim 9, wherein the compound is eucalyptol.

16. A method of preparing a composition having antimicrobial activity comprising extracting a plant material in an organic solvent,

contacting the extracted material with a chromatographic separation system, and

eluting the chromatographic separation system with a mobile polar phase to obtain a composition,

wherein the plant material is from *Mammea Americana, Marchantaceae polymorpha,* or *Callistemon citrinus,* and wherein the composition has antimicrobial activity.

17. The method of claim 16, wherein said plant is *Mammea Americana mamey Amarillo.*

18. The method of claim 16, wherein said plant is *Marchantaceae polymorpha hepatica.*

19. The method of claim 16, wherein said plant is *Callistemon citrinus skeels.*

20. The method of claim 17, wherein said composition comprises at least one compound selected from the group consisting of cobaltacene-octgomet, or stigmastan-3,5,-diene.

21. The method of claim 18, wherein said composition comprises α-caryophylene, β-caryophylene, and caryophylene oxide.

22. The method of claim 19 wherein said composition comprises at least one compound selected from the group consisting of galoxolide, benzyl salicylate, or α-pinene.

23. The method of claim 16, wherein the organic solvent is methylene chloride.

24. The method of claim 16, wherein the antimicrobial activity is against a mycobacterium.

25. The method of claim 24, wherein the mycobacterium is *M. avium, M. bovis, M. intracellulare, M. kansaii, M. leprae, M. marinum, M. phlei, M. scrofulaceum, M. smegmatis, M. fortuitum, M. tuberculosis,* or *M. ulcerans*.

26. A method of inhibiting the growth of a mycobacterium, comprising administering a composition comprising a carrier and at least one compound selected from among cobaltacene-octgomet, stigmastan, 3,5-diene, galoxolide, benzyl salicylate, eucalyptol, and α-pinene.

27. The method of claim 26, wherein said mycobacterium is *M. avium, M. bovis, M. intracellulare, M. kansaii, M. leprae, M. marinum, M. phlei, M. scrofulaceum, M. smegmatis, M. fortuitum, M. tuberculosis,* or *M. ulcerans*.

28. The method of claim 26, wherein said mycobacterium is in a mammal and said mammal is a human or a bovine.

29. The method of claim 28, wherein said composition is administered orally.